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GRANT NUMBER: DAMD17-94-J-4217

TITLE: Progression and Metastasis of Mammary Carcinomas: Potential Role of the Mucl Glycoprotein

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REPORT DATE: August 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

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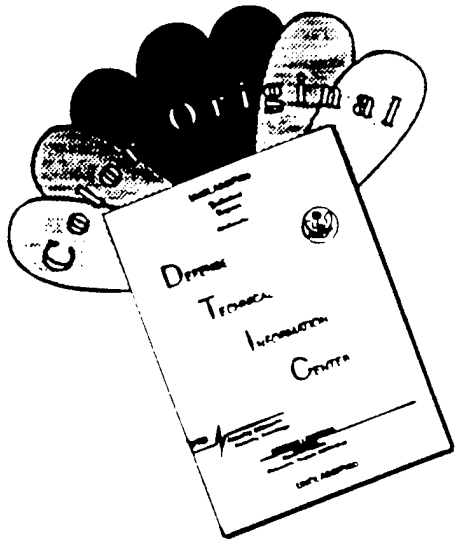

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Introduction:

Our goal is to understand the function of the tumor-associated mucin, MUC1, in the progression of cancer in the mammary gland. MUC1 is highly expressed by the majority of cancers and, in particular, by >92% of primary and metastatic breast cancers. The MUC1 protein is a large, rod-like molecule that projects far from the cell surface as a long filament. The protein core is extensively glycosylated through O-glycosidic linkage to serine and threonine, with as much as 50 to 90% of its molecular mass made up of oligosaccharide side chains. This contributes significantly to the rigidity of the molecule. MUC1 is expressed on normal epithelial tissues at low levels. Appearance of MUC1 correlates closely with epithelial differentiation in various organs and is detected well before the organs are functional. The presence of the large, highly extended molecule of MUC1 on the surface of epithelia suggests that it may act as a physical barrier protecting the cells. MUC1 may be involved in epithelial morphogenesis, perhaps acting to mask adhesive molecules present on the cell surface and aiding in the formation of a lumen. When epithelial tissues become cancerous, MUC1 expression is increased at least ten fold, and the glycosylation and spatial distribution of the protein at the cell surface are altered. MUC1 in normal polarized epithelia is expressed only at the apical side of lumens and ducts. However, in many adenocarcinomas polarization is lost, and the protein is found over the entire surface of the cells. Our hypothesis is that expression of this protein benefits tumor cells and their metastatic counterparts, perhaps by altering the adhesive properties of cells or by providing a protective layer around cells that may shield them from immune surveillance.

The ability to create mice that possess deficiencies in specific genes is providing important insights into the physiological role played by specific proteins during embryonic and postnatal development and during adult life. The expression pattern of the *Muc-1* gene in the adult and embryo of the mouse is similar to that of the human (the human gene designation is *MUC1*; the mouse gene is *Muc-1*) (Braga et al., 1992). Muc-1 expression is also elevated in mouse mammary gland tumors. Since mammary gland cancer in the mouse closely resembles human breast cancer and expression patterns are similar, our experiments should enable us to analyze the functional role of Muc-1 in the development and progression of cancer. To investigate the biological function of the Muc-1 protein we disrupted the *Muc-1* gene using homologous recombination in mouse embryonic stem cells. Mice were generated that lacked expression of the Muc-1 protein. We and others had postulated that Muc-1 on the apical surface of differentiating epithelial cells may repel adjacent cells or mask adhesive molecules, thus promoting the formation of a lumen. However, we were surprised to find that, despite the

widespread expression of Muc-1 during epithelial organogenesis, mice lacking Muc-1 protein were born at the expected frequency and appeared normal in all respects.

Direct evidence of a role for MUC1 in the development and progression of breast cancer has not been demonstrated previously. In many cancer cells polarization of the epithelial cells is lost and the MUC1 protein can be detected on all cell surfaces, including those facing the stroma and adjacent cells. Under these circumstances, the anti-adhesive property of MUC1 may destabilize cell-cell and cell-substratum interactions, thus promoting the disaggregation of a tumor site, leading to tumor spread and metastasis. Previous studies have suggested various possible roles for the MUC1 mucin in facilitating tumor growth, including inhibition of cell-cell contacts, protection from recognition and destruction by immune cells, and also serving as an E-selectin ligand to facilitate escape of metastatic cells from the blood stream. Thus overexpression of the Muc-1 molecule could provide many potential benifits to tumor cells.

We are currently using Muc-1 deficient and control mice to investigate the role of the Muc-1 molecule in normal development and in the development and progression of breast cancer in mice. The following progress was achieved in year one of the study:

Mice homozygous for the *Muc-1* mutation have been bred onto inbred 129SV and C57Bl/6J (N8) lines and have been demonstrated to be healthy, fertile and viable. In all cases, animals homozygous for the disrupted *Muc-1* allele (-/-) were obtained at the expected Mendelian frequency of 1:2:1 (Wild type : Heterozygotes : Homozygous Mutants). Loss of Muc-1 expression did not appear to effect organogenesis as examination of hematoxylin-eosin stained sections prepared from all the major organs revealed no obvious differences between Muc-1 deficient mice and their corresponding litter mates. Muc-1 deficient mice failed to demonstrate significant upregulation of expression of mucin-like genes or membrane glycoproteins including Muc-2, Muc-4, ASGP-2, CD34, CD43 (leukosialin), glycophorin and MadCAM-1.

We have demonstrated for the first time that the Muc-1 molecule facilitates the growth of breast tumors in mice transgenic for the polyoma virus middle T antigen. Although similar numbers of mice developed tumors by 4 months of age in both Muc-1 deficient and control groups, tumor growth rate was significantly decreased in mice homozygous for the *Muc-1* mutation when compared with wild type control mice. In addition, there was a trend towards decreased lung metastasis in Muc-1 deficient mice. Overall, 58% of mice developed grossly observable lung metastases, with 53% of *Muc-1* -/- mice and 67% of *Muc-1* +/+ mice developing

metastases. Based on the sample sizes in this study, the power to statistically detect the observed difference was only 33%. It is possible that with a larger sample size, this difference in metastatic rate would be statistically significant.

Overall studies to date have demonstrated a role for Muc-1 in facilitating the growth of breast cancer induced by the polyoma virus middle T antigen, but they have failed to demonstrate a role for Muc-1 in the development or functioning of normal epithelial glands. We have continued to investigate the role of Muc-1 in organogenesis, mammary tumor development and metastasis.

Methods and Materials:

Mammary Gland Whole Mount Analysis: Muc-1 deficient and wild type mice (n= 3 per group per time point) were terminated at 3, 5, 7 and 14 weeks of age. Auxiliary and inguinal mammary glands were removed and half were fixed in methacarn, parafin embedded, sectioned at 5 uM and stained with hematoxylin and eosin for histological examination. The other halves were spread out on glass slides and fixed in acetone overnight, the tissues were subsequently cleared by passage through xylene and a graded series of alcohols (100, 95, 80,70 and 40%). The tissues were subsequently stained for 20 min in Mayer's hematoxylin and destained in ammonium water (3.4mM NH_4OH).

Cell Proliferation and Apoptosis: To study cell proliferation rates and the rate of apoptosis, 20 female mice transgenic for the polyoma virus middle T antigen were produced on the Muc-1 deficient or wild type backgrounds (n= 10 per group). For the experiment, mice were palpated twice weekly from day 60 and were terminated when the tumors reached a weight of 1 gram. On termination day, mice were injected i.p. with 1.5 mg/mouse of 5' Bromodeoxyuridine (BrdU, Sigma, St. Louis, MO). Mice were terminated 2 h post injection and tumors were removed, fixed in methacarn for 2 h and stored in 70% ethanol at 4°C. Tumors were parafin embedded and 5uM serial sections were cut.

To measure cell proliferation, tumors were stained for BrdU incorporation. Slides were deparaffined, treated with a mild enzymatic digest (0.005% pepsin in 0.025M HCl for 15 min at RT) then blocked in 50% fetal calf serum (Gibco, Grand Island, NY). The DNA was denatured by treatment with 2N HCl (60 min at 37°C). Sections were treated with anti-bromodeoxyuridine primary antibody (Boehringer Mannheim, Indianapolis, IN) followed by a peroxidase-conjugated anti-mouse secondary antibody (Dako, Carpinteria, CA). The tissues were then exposed to diaminobenzoic acid for approximately 1 to 2 minutes and counterstainded with hematoxylin.

Cells staining positive for BrdU and total cells were enumerated for 5 randomly chosen fields at 400X magnification using a 25 square 1mm2 reticule. Control sections treated with an irrelevant primary antibody failed to exhibit significant nuclear staining for any of the tissues tested. Liver sections exhibited proliferation rates of less than 1%.

To measure cellular levels of apoptosis and necrosis, tumor sections were stained using the TUNEL assay. Slides were deparaffined, treated with a mild enzymatic digestion (0.004% pepsin in 0.025M HCl for 15 min at RT) and stained with the in situ cell death detection kit (Boehringer Mannheim). The level of apoptosis in each specimen was rated on a 4 point scale and all observations were confirmed by two independent observers. Liver sections did not exhibit significant levels of apoptosis. Similarly, tumor sections labeled in the absence of the terminal deoxynucleotide transferase enzyme failed to stain for apoptosis.

In Vitro Tumor Proliferation: To investigate the ability of tumor cells to proliferate under conditions in which oxygen and nutrient concentrations were not growth limiting, cells from middle T antigen induced mammary tumors growing in Muc-1 deficient and wild type mice were placed into long term tissue culture. For the growth rate experiments, cells were plated in 6 well plates at a concentration of 5 X 10^4 cells per well in DMEM containing 10% fetal calf serum. The cells were incubated at 37°C, 10% CO_2 and the media was changed every 4 days. To evaluate cell proliferation, cells were detached from the dish by a 5 min treatment with versine at 37°C followed by a 2 min exposure to trypsin at 37°C. Cells were counted on a hemocytometer on a phase contrast microscope. Cell counts were performed in triplicate and the experiment was repeated 3 times with similar results.

Results:

Specific Aim 1: Analysis of the effects of Muc-1 gene mutations on epithelial organogenesis.

Our previous studies failed to indicate a loss of viability or defects in the organs of mature Muc-1 deficient mice. However, it is possible that subtle defects in the organogenesis may still occur. To investigate this possibility, mammary gland organogenesis in Muc-1 deficient and control animals was studied. The mammary gland is an organ system which develops late, maturing in the juvenile mouse and it is not required for survival of the individual. Thus it is an excellent system in which to investigate possible subtle changes in organogenesis in Muc-1 deficient mice. To study the role of Muc-1 in the development of the mammary gland, virgin female 129SV mice of the Muc-1 deficient and wild type strains were terminated at 3, 5, 7

and 14 wk of age. Inguinal and auxiliary mammary glands were removed and prepared for whole mount staining or for sectioning. Ductal end buds were just beginning to invade the mammary fat pads at 3 wk of age and Muc-1 deficient and wild type mice did not appear to differ in the extent of invasion (figure 1). However, by 7 wk of age, when the ductal elements filled approximately half of the mammary fat pad in wild type mice, Muc-1 deficient mice had greater penetration of ductal elements into the fat pad and an apparent increase in the density of ductal elements (figure 1). At 14 wk mammary glands of Muc-1 deficient mice demonstrated increased branching of ducts compared with wild type mice. Thus it appears that ductal elements in the developing mammary glands of Muc-1 deficient mice have a proliferation advantage over wild type mice during the period in which the ductal elements grow through the mammary fat pad. However, this data is preliminary and a more extensive study is being planned to investigate this effect of Muc-1 deficiency. It is possible that the increased branching of mammary gland ducts in Muc-1 deficient mice is related to the stage of estrus cycle of the mouse rather than the lack of Muc-1 expression. To control for this possibility, in future studies control and Muc-1 mutant animals will be housed together to allow for synchronizing of estrous phase and animals will be staged.

Specific Aim 2: Analysis of the effects of Muc-1 gene mutation on tumor formation and progression.

We have demonstrated that overexpression of Muc-1 facilitates tumor growth of polyoma virus middle T antigen induced mammary tumors. However, the mechanism by which Muc-1 overexpression facilitates tumor growth is not clear. It is possible that Muc-1 overexpression may increase the rates of cellular proliferation or decrease rates of cell death. An effect of Muc-1 on rates of cell proliferation and/or death could be the result of Muc-1 overexpression blocking cell-cell contact and thus preventing the transmission of contact mediated growth inhibitory signals from surrounding normal cells. It has been demonstrated that MUC1 overexpression in cell lines can block cell-cell and cell-substratum contacts (Ligtenberg et. al., 1992; Wesseling et. al., 1995). Alternately, Muc-1 may be involved in signal transduction in tumor cells. The cytoplasmic tail of Muc-1 is 88% conserved in all mammalian species tested and it contains 7 tyrosine residues, 6 of which are conserved (Spicer et. al., 1995). Recently it has been demonstrated that MUC1 is phosphorylated *in vivo* on tyrosine and serine residues and that phosphorylated MUC1 may bind signal transduction molecules through SH2 domains (Zrihan-Licht et. al., 1994; Pandey et. al., 1995).

To investigate the role of Muc-1 overexpression in tumor cell growth, we measured rates of cellular proliferation, apoptosis and levels of necrosis in tumors from Muc-1 deficient and

wild type control mice. Further, to ensure that nutrient or oxygen levels within the tumor were not limiting growth rate of tumor cells, rates of proliferation of tumor cells derived from polyoma virus middle T antigen induced mammary tumors growing in Muc-1 deficient and wild type mice were measured *in vitro*. There were no differences in rates of cellular proliferation (Figure 2), apoptosis or levels of necrosis (Figure 3) between mammary tumors from Muc-1 deficient and wild type control mice. Further, *in vitro* rates of cellular proliferation did not differ significantly between mammary tumors from Muc-1 deficient and wild type control mice (Figure 4).

To insure that the effect of Muc-1 overexpression was not specific to tumors induced by the polyoma virus middle T antigen oncogene, a study was initiated to investigate the effect of Muc-1 gene mutation on tumor development and progression induced by the neu protooncogene. The neu transgenic mouse is a useful model for several reasons. It is estimated that 30 percent of human breast cancers overexpress the neu protooncogene (Slaman et. al., 1987). Further, neu proto-oncogene transgenic mice have long tumor latencies and develop focal mammary adenocarcinomas surrounded by hyperplastic mammary epithelium similar to that observed in humans (Guy et. al., 1992a&b).

A study was initiated to investigate the effect of *Muc-1* gene mutation on tumor development and progression induced by the neu protooncogene. Female neu transgenic mice homozygous for the Muc-1 mutation or wild type Muc-1 genes were produced (n = 80 per group). These animals were outbred mice resulting from two crosses of FVB neu transgenic mice on to C57Bl/6 Muc-1 deficient or wild type control mice. To ensure that B6 mice do not contain genes which could alter the growth of the neu proto-oncogene induced mammary tumors, neu transgenic mice were also backcrossed on to B6 mice and tumor growth was compared with that seen in inbred FVB mice. Unfortunately, tumor growth was significantly altered in FVB/B6 F1 mice (Figure 5). While inbred virgin FVB female mice begin to develop tumors at about 7 months of age and have a 65% tumor incidence by 12 months of age, FVB/B6 F1 mice showed a marked delay in tumor initiation and tumors were not observed in F1 mice until 18 months of age. The F1 mice had a 5% tumor incidence at 18 months of age (2/50). This delay in tumor incidence suggests that a dominant acting gene(s) present in the B6 genome can modulate the growth or induction of neu induced mammary tumors. Clearly, the effect of the B6 genome on neu proto-oncogene induced mammary tumor growth would mask any effect of Muc-1 overexpression on tumor growth. However, as overexpression of the neu proto-oncogene is observed in 30% of human breast cancers, other genetic loci which could effect tumorigenesis by the neu gene are clearly of interest. To study this phenomena further, FVB/B6 F1 mice were

backcrossed on to inbred FVB mice to produce mice with 75% of their genome of the FVB genotype and tumor growth was studied in virgin neu positive female offspring. A small fraction of these mice began to develop tumors by 7 months of age and by 14 months of age approximately 35% of these mice developed tumors. This finding suggests that the effect of the B6 genome on neu proto-oncogene induced mammary tumorigenesis is likely due to one or a small number of dominantly acting genes. We are currently investigating the chromosomal localization of these genes using genetic linkage analysis by microsatellite polymorphism analysis using CA dinucleotide repeats.

Specific Aim 3: Analysis of the effect of Muc-1 gene deletion on metastasis of mammary gland tumors.

The failure of neu proto-oncogene induced tumors to grow in FVB/B6 F1 mice prevented an analysis of the effect of Muc-1 overexpression on metastasis in this system. Current efforts to study this phenomena will focus on the ability of polyoma virus middle T antigen induced tumor cells isolated from Muc-1 deficient and wild type control mice to seed to the lungs of Muc-1 deficient and wild type control mice following tail vein injection.

Conclusions and Future Studies:

We have demonstrated that mice homozygous for the Muc-1 mutation are healthy, fertile and do not exhibit any deleterious effects in two different strains of mice. The present studies indicate that *Muc-1* mutation alters the organogenesis of the mammary gland. It is not yet clear how Muc-1 expression affects development of the mammary gland. It is possible that other transmembrane or extracellular molecules are upregulated to compensate for the lack of Muc-1 in the developing mammary gland and that these molecules stimulate increased proliferation of the ductal elements. Our studies have failed to indicate upregulation of mRNA for other known mucin like genes. However, it remains possible that other molecules may be upregulated to compensate for the lack of the *Muc-1* gene. Alternately, Muc-1 could have a suppressive influence on the proliferation of mammary ductal epithelial elements.

These studies demonstrate for the first time that the Muc-1 molecule facilitates growth of breast tumors in mice transgenic for the polyoma virus middle T antigen. Tumor growth rate was significantly decreased in mice homozygous for the Muc-1 mutation when compared with wild type control mice. Interestingly, the tumors did not exhibit different rates of proliferation or necrosis as measured by BrdU incorporation or in vitro proliferation. These findings suggest that

facilitation of tumor growth induced by the overexpression of the Muc-1 molecule by mammary tumor cells does not involve large changes in rates of cellular proliferation or apoptosis. However, it is possible that small changes in the rates of cellular proliferation and/or apoptosis which are below the sensitivity of the current assay system to detect could result from the overexpression of Muc-1 by mammary tumor cells. Such subtle changes in cell growth or death rates could be sufficient to account for the observed differences in tumor growth observed in this model given the exponential nature of tumor growth.

Another potential mechanism by which Muc-1 overexpression could affect tumor development and metastasis is by modulating the immunogenicity of mammary tumor cells. It has been demonstrated that the overexpression of MUC1 *in vitro* by transfecting tumor cells to express high levels of MUC1 protein results in protection from lysis by natural killer cells and cytotoxic T lymphocytes (Wiel-van Kemenade et. al., 1993). It has been speculated that the presence of the large negatively charged MUC1 molecule on the tumor cell surface blocks access of immune cells to tumor specific antigens and other cell adhesion molecules present on the tumor cell surface. Thus it is possible that overexpression of Muc-1 by mammary tumor cells facilitates tumor growth by blocking tumor recognition and lysis by natural killer cells and cytotoxic T lymphocytes. To test this hypothesis, we will investigate mammary tumor growth rate and metastasis in Muc-1 deficient and wild type mice that are immunocompromised by the lack of either T lymphocytes or natural killer cells. To obtain mice lacking T lymphocytes the Rag-1 knockout mice will be used (Mombaerts et. al., 1992). These mice lack a gene, the recombinase activating gene 1, required to recombine the variable domains of immunoglobulins and T cell receptors. As a result T and B lymphocytes are not functional in these mice. This genotype is currently being bred on to polyoma virus middle T antigen transgenic mice containing the wild type or the mutant *Muc-1* gene. For the study, 4 groups of mice will be utilized. All mice will be virgin females containing the polyoma virus middle T antigen transgene. Muc-1 deficient and wild type mice will either have the wild type Rag-1 genotype or be homozygous for the mutant Rag-1 gene. Chi-square analysis suggests that 40 females will be required for each condition. Tumor growth will be followed for 120 days by palpation and then mice will be terminated and the rate of lung metastasis evaluated.

To investigate whether Muc-1 overexpression is required to block natural killer cell recognition and lysis of mammary tumor cells, natural killer cells will be deleted from mice by the repeated injection of the monoclonal antibody NK1.1 (Seaman et. al., 1987). Preliminary experiments demonstrate that interperitoneal injections of the antibody every 7 days will be sufficient to maintain complete depletion of natural killer cells. Four groups of mice will be used

in this study as discussed for the Rag-1 mutant mice. These studies will allow an elucidation of the role of natural killer cells and cytotoxic T lymphocytes in this tumor model.

To insure that the role of Muc-1 overexpression in facilitating tumor growth is not specific to tumors induced by the polyoma virus middle T antigen oncogene, we attempted to study the role of Muc-1 expression in mammary tumors induced by the overexpression of the neu proto-oncogene. Unfortunately, the neu transgenic mice were produced on the FVB background and our studies revealed that C57Bl/6 mice contain one or several genes that act in a dominant fashion to suppress tumor initiation by the neu proto-oncogene. This finding is similar to that seen in min mice, where the mom locus (modifier of min) suppresses tumorigenesis in a dominant fashion (Dietrich et al., 1993). As the overexpression of the neu proto-oncogene is observed in 25-30% of human breast cancer patients, the identification of genes that affect the ability of the neu gene to induce mammary tumors is of great interest. We are currently investigating the chromosomal location of these modifier loci using linkage analysis in mice which are 75% FVB and 25% C57Bl/6. Approximately 1/3 of these mice develop mammary tumors in the same time frame as pure strain FVB mice. We are currently genotyping this subset of mice to identify loci that are consistently homozygous for the FVB alleles. These regions of the chromosome are candidates to contain genes which may modify the tumorigenesity of the neu proto-oncogene.

Studies to investigate the role of Muc-1 overexpression in the development and progression of mammary tumors induced by the overexpression of the neu proto-oncogene in the breast will be rescued by breeding the Muc-1 deficient mice back onto the FVB strain. However it will require 12 backcrosses to achieve Muc-1 deficient mice that are of an inbred FVB strain. Such a project will require 2 years of backcrossing plus an additional 18 months to study the effect of Muc-1 deficiency on neu induced mammary tumors using these animals. While this project is currently being initiated in our lab, it is clearly beyond the scope of the current proposal to complete. Also, although time does not permit an investigation of the effect of Muc-1 deficiency in other tumor models in the current proposal, our lab will investigate the effect of Muc-1 deficiency using a chemical carcinogenesis model of mammary tumor induction in which tumors are induced by the injection of MNU + MPA. The combination of these 3 experimental systems should indicate if Muc-1 plays an important role in the development or progression of breast cancer.

Interestingly, although more Muc-1 deficient mice developed lung metastases that did wild type mice, this trend towards decreased rates of tumor metastasis in Muc-1 deficient mice

did not reach statistical significance. It is possible that the rapid kinetics of the middle T tumor phenotype may have rendered some immune responses impotent. The middle T mice exhibit hyperplastic mammary glands as early as three weeks of age and the rapid production of multifocal mammary adenocarcinomas. In many animals tumors developed in every mammary gland. To address questions regarding the sensitivity of the assay to detect differences in metastatic abilities, a second assay of metastatic ability will also be utilized. Tumor cell lines have been isolated from polyoma virus middle T antigen induced mammary tumors growing in Muc-1 deficient and wild type mice. These cell lines and freshly isolated tumor cells will be injected into the tail veins of Muc-1 deficient and wild type mice and the lung colonizing ability of these cell lines compared as well as the longevity of tumor cells in the bloodstream. This tumor metastasis system has the advantage of controlling for the seeding ability of tumor cells and focuses on the ability of tumor cells to arrest in the target organ, extravasiate from the bloodstream and grow in the target organ. These studies should help to define whether or not overexpression of the Muc-1 molecule facilitates tumor metastasis.

The studies described in this report utilize the unique strength of the *Muc-1* mutant mouse model to investigate the role of the Muc-1 molecule in organogenesis, tumor development and progression and in tumor metastasis. These are the first studies to directly demonstrate a role for Muc-1 overexpression in facilitating the growth of breast cancer *in vivo*. It is hoped that in the long term the data derived from these studies could be used to improve the treatment of human breast cancer.

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Figure Legends:

Figure 1. Mammary Gland Organogenesis in Muc-1 Deficient and Wild Type Mice.
Muc-1 deficient and wild type virgin female mice (n=2-3 per group) were terminated at 3, 5, 7 and 14 wk. Mammary glands were removed, spread on glass slides, fixed in acetone overnight and stained with Mayer's hematoxylin. Equivalent auxillary mammary glands were viewed and photographed under identical conditions. Ductal elements begin to invade the mammary fat pad around 3 wks of age and Muc-1 deficient and control mice did not differ in the extent of infiltration at this time. By 7 wk, Muc-1 deficient animals showed an increase in the extent of ductal invasion of the mammary fat pad and in the branching of the ductal elements relative to control mice. By 14 wk, ductal elements filled the mammary fat pads of both Muc-1 deficient and control mice, but Muc-1 deficient mice showed increased branching of ductal elements relative to wild type control mice.

Figure 2. Rates of Proliferation of Cells in Mammary Tumors From Muc-1 Deficient and Wild Type Mice.
Muc-1 deficient and wild type virgin female mice (n=10 per group), transgenic for the polyoma virus middle T antigen were terminated when tumors reached a weight of 1 gram. On termination day, mice were injected with bromodeoxyuridine 2 h prior to termination, tumors were removed, fixed in methacarn, paraffin embedded and sectioned for immunohistochemistry. Tumor sections were stained for incorporation of BrdU using an anti-BrdU antibody and positive cells imaged with a horse radish peroxidase conjugated secondary antibody followed by incubation with diaminobenzoic acid. Cells positive for BrdU incorporation and total cells were enumerated in 5 randomly chosen fields at 400X magnification using a 25 square $1mm^2$ reticule for each tumor. There were no detectable differences in rates of tumor cell proliferation between Muc-1 deficient and wild type mice.

Figure 3. Relative Degree of Apoptosis and Necrosis in Mammary Tumors From Muc-1 Deficient and Wild Type Mice.
Tumor sections (from animals described in Figure 2) were stained using the TUNEL assay. The level of apoptosis and necrosis in each specimen were rated on a 4 point scale and all observations were confirmed by two independent observers. Mammary tumors induced in Muc-1 deficient and wild type mice did not differ in the levels of apoptosis or necrosis present.

Figure 4. Proliferation of Muc-1 Deficient and Wild Type Mammary Tumor Cells In Vitro.
Mammary tumors developing in Muc-1 deficient and wild type mice transgenic for the polyoma virus middle T antigen were placed in long term culture. Tumor cells were plated in 6 well plates at 5×10^4 cells/well. At various times post plating, tumor cells were dissociated from dishes and counted on a hemocytometer under phase contrast. In three separate experiments, tumor cells which did not express the Muc-1 protein grew equally as well as tumors from wild type mice which express the Muc-1 protein. Cell counts for each time point were performed in

triplicate and standard deviations did not exceed 10%. Tumors from wild type mice were shown to express Muc-1 under the culture conditions used in this study.

Figure 5. Tumor Incidence in Neu Proto-Oncogene Transgenic Female Mice

Inbred FVB virgin female mice transgenic for the neu proto-oncogene developed mammary tumors between 7 and 12 months of age with a 70% incidence at 12 months of age. In contrast, at 18 months of age only 5% of FVB x C57Bl/6 F1 transgenic females developed mammary tumors. When F1 mice were backcrossed onto inbred FVB mice (F1B1), neu transgenic females developed mammary tumors with approximately a 35% incidence at 14 months.

FIGURE 1

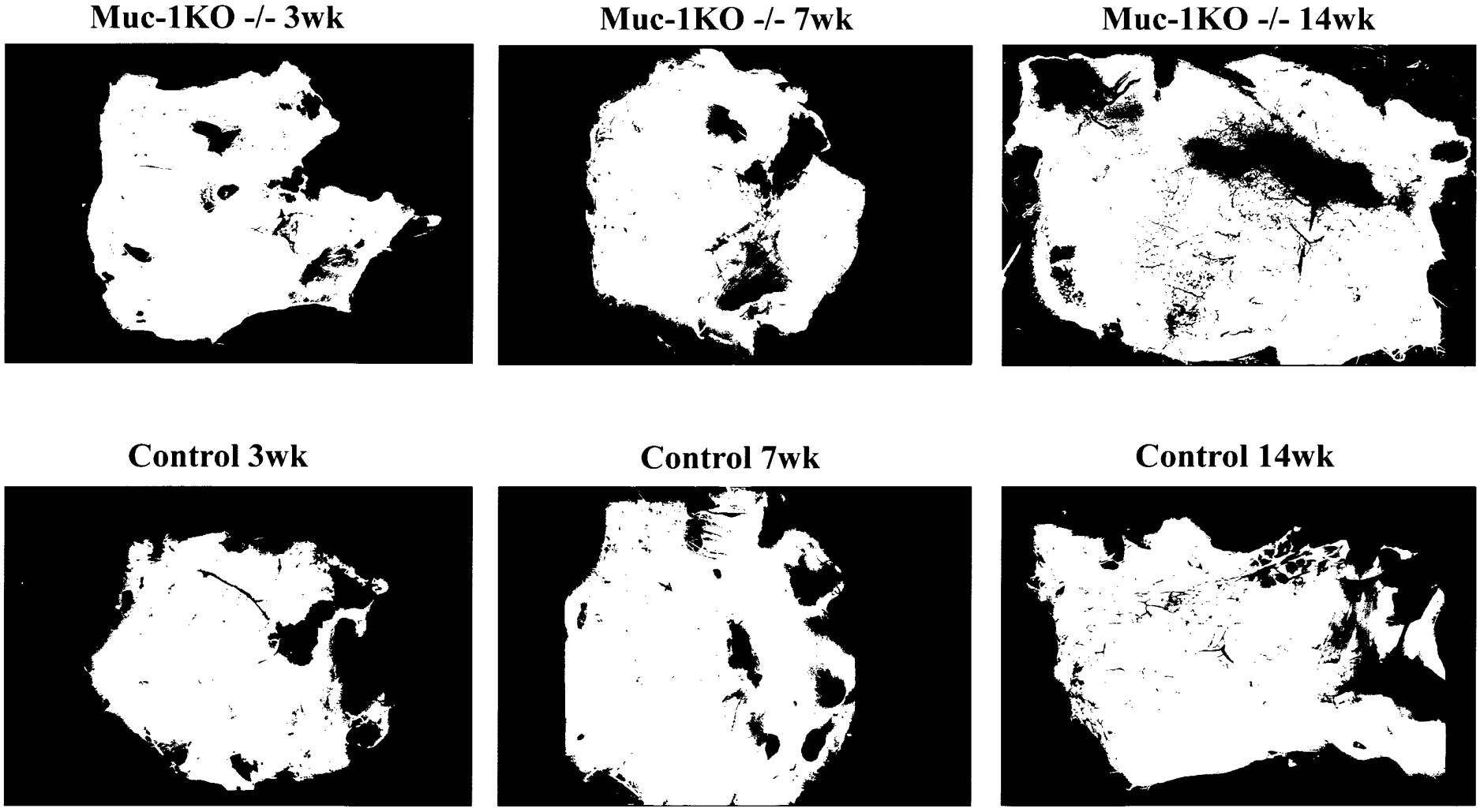

Muc-1KO -/- 3wk
Muc-1KO -/- 7wk
Muc-1KO -/- 14wk
Control 3wk
Control 7wk
Control 14wk

Figure 2.

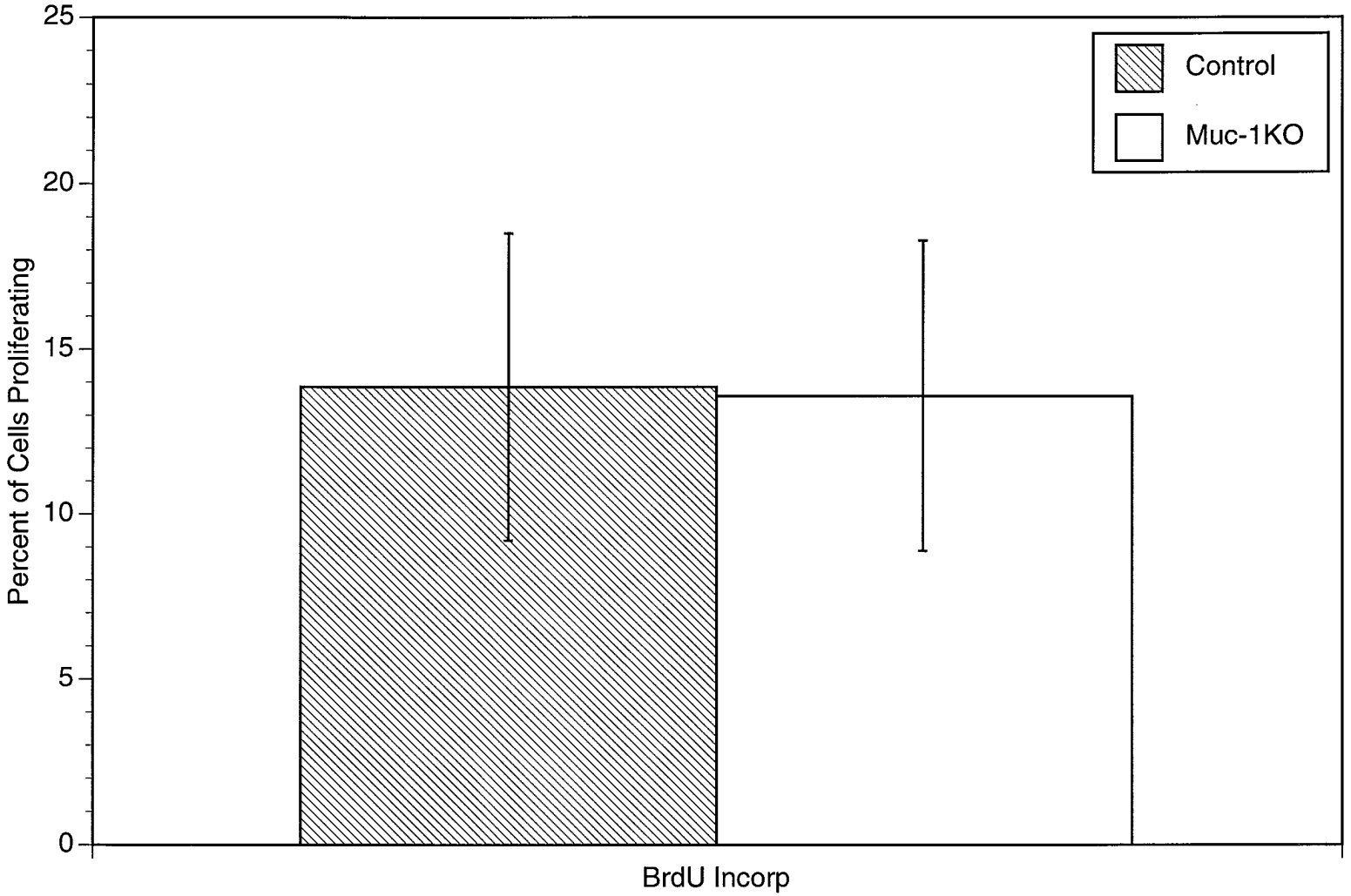

25
20
15
10
5
0
Percent of Cells Proliferating
Control
Muc-1KO
BrdU Incorp

Figure 3.

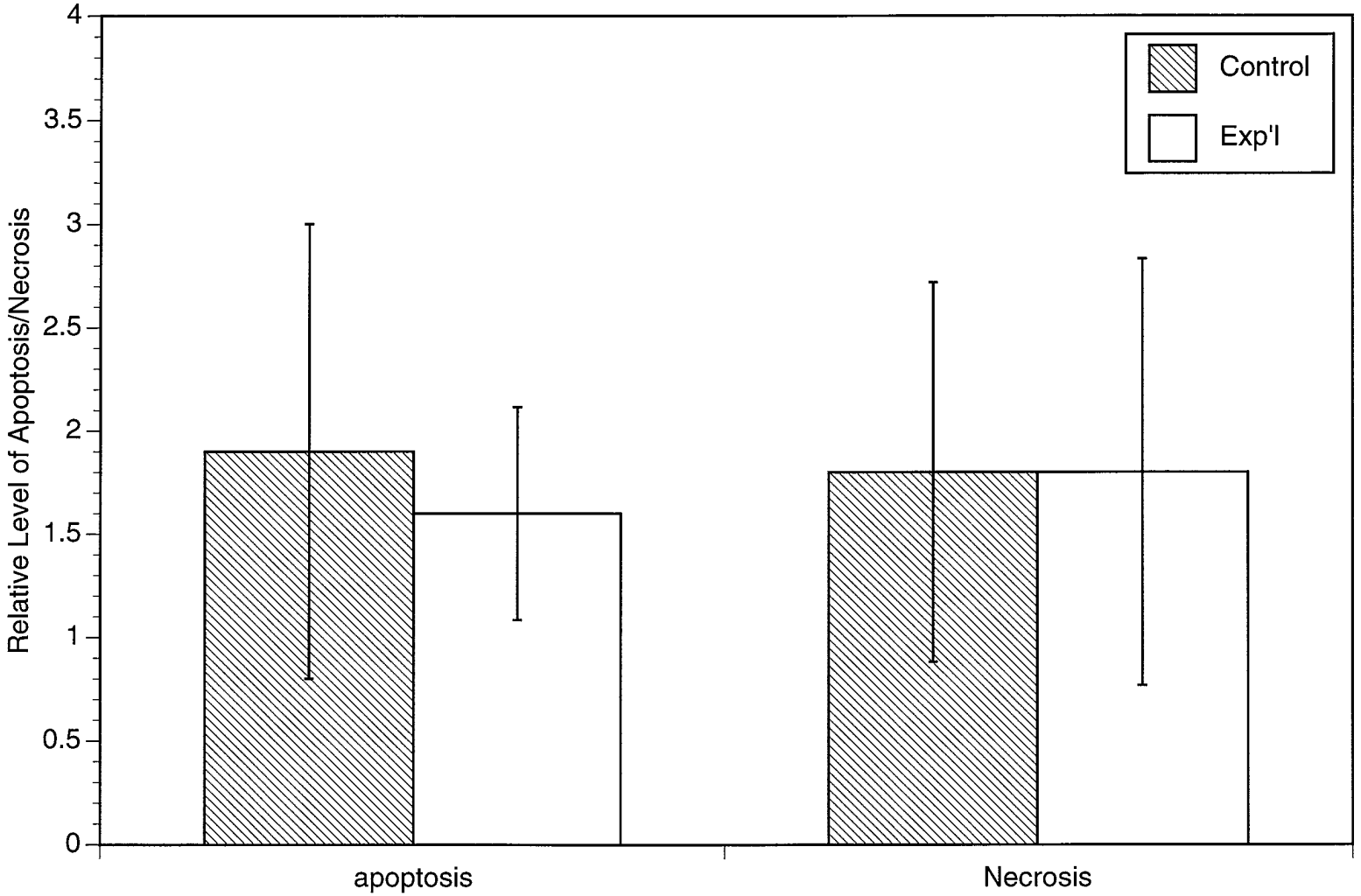

Control
Exp'l
Relative Level of Apoptosis/Necrosis
4
3.5
3
2.5
2
1.5
1
0.5
0
apoptosis
Necrosis

Figure 4.

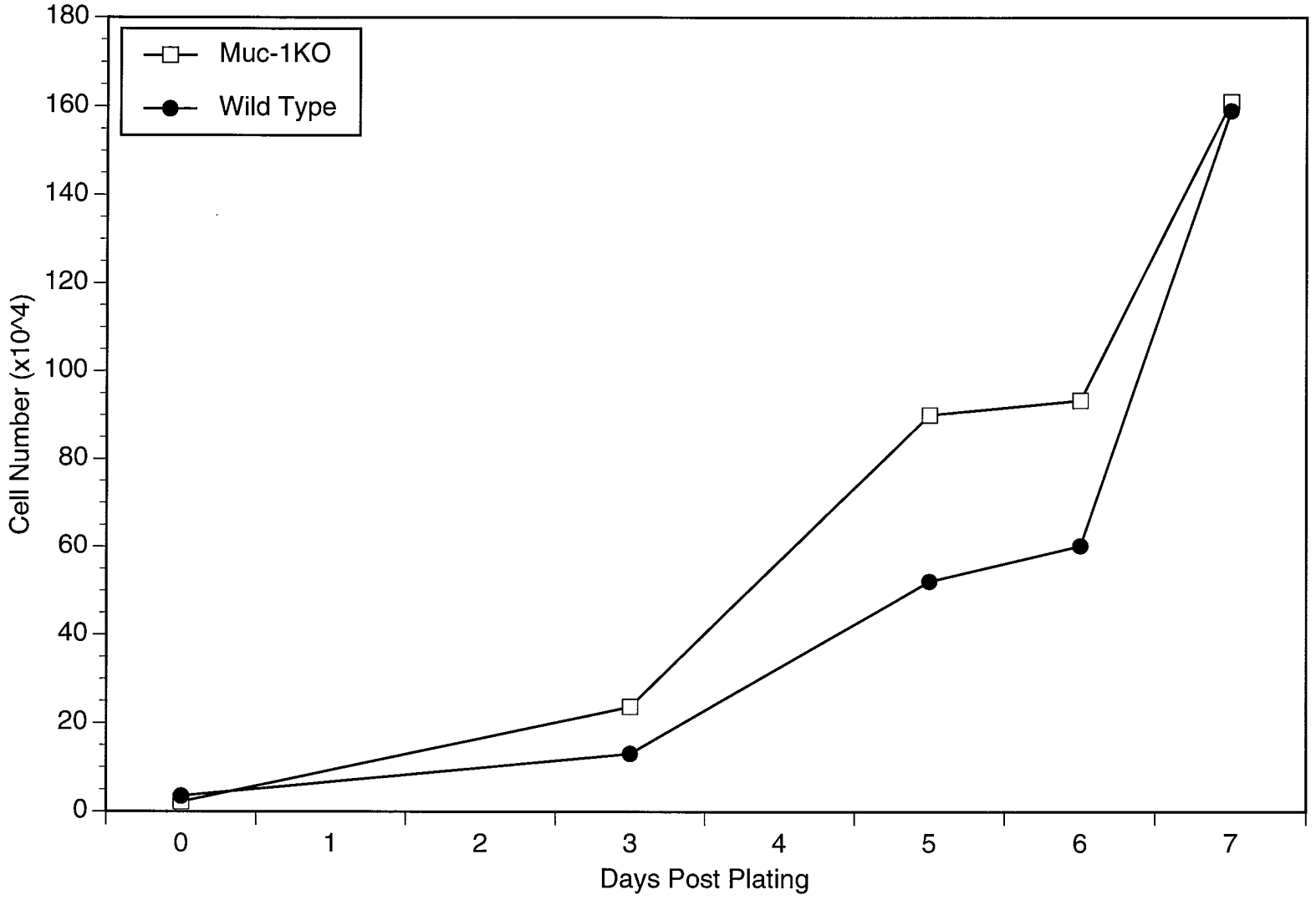
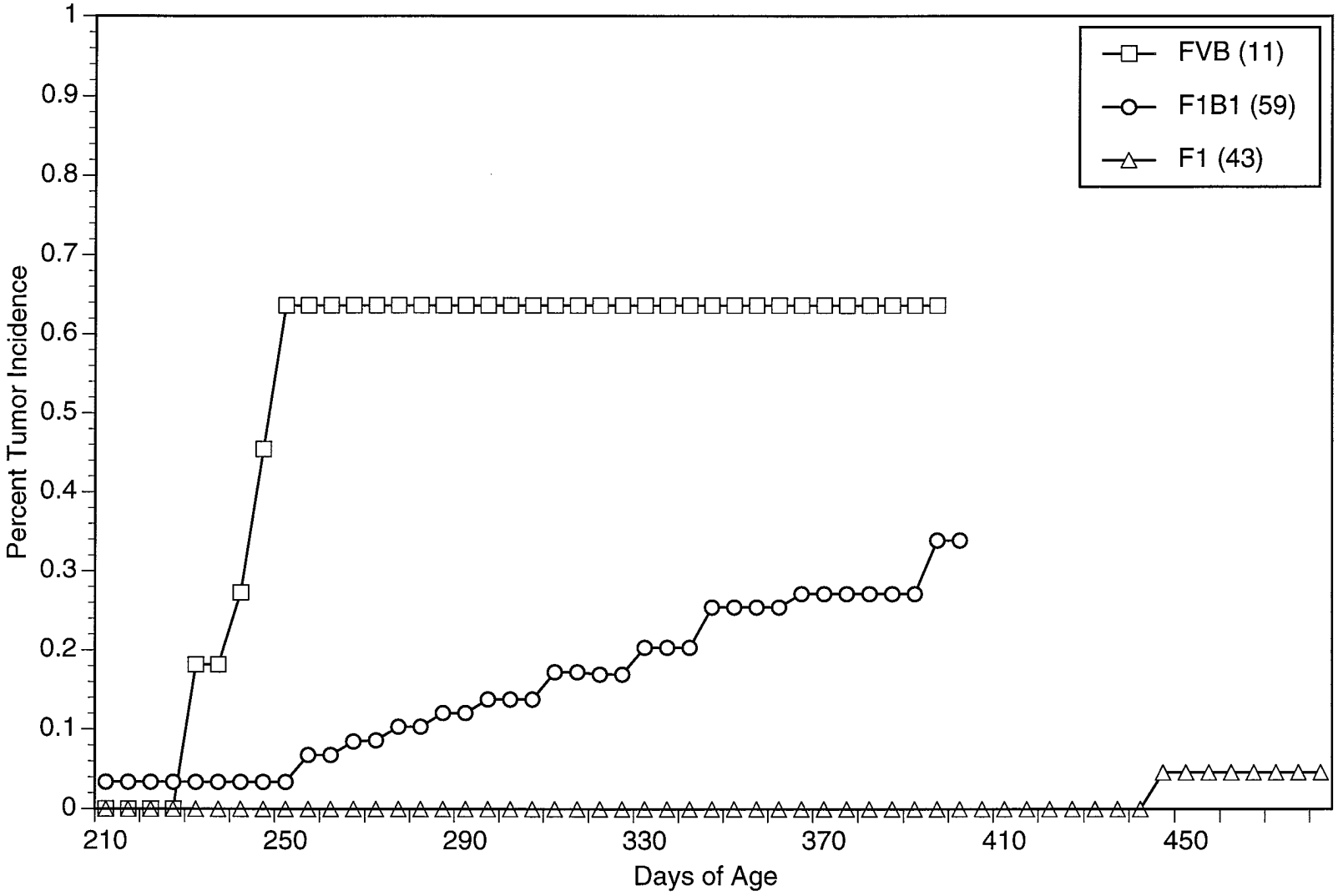

Muc-1KO
Wild Type
Cell Number (x10^4)
180
160
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
Days Post Plating

Figure 5.


FVB (11)
F1B1 (59)
F1 (43)
Percent Tumor Incidence
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
210
250
290
330
370
410
450
Days of Age